AD-A062 984　ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC　F/G 7/2
ALTERED ZINC HOMEOSTASIS AND HEPATIC ACCUMULATION OF METALLOTHI--ETC(U)
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Altered Zinc Homeostasis and Hepatic Accumulation of

Metallothionein in Indomethacin-Induced Enteropathy

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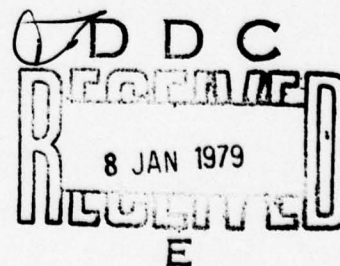P. Z. SOBOCINSKI, G. L. KNUTSEN, W. J. CANTERBURY, JR.,

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Running title: INDOMETHACIN AND ZINC HOMEOSTASIS

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Altered Zinc Homeostasis and Hepatic Accumulation of Metallothionein in Indomethacin-Induced Enteropathy.		5. TYPE OF REPORT & PERIOD COVERED Interim rept.
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) P. Z. Sobocinski, G. L. Knutsen, W. J. Canterbury, Jr. E. C. Hauer		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS U.S. Army Medical Research Institute of Infectious Diseases, SGRD-UIP-S Fort Detrick, Frederick, MD 21701		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A 3M161102BS03 00 013
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command, Office of the Surgeon General Department of the Army, Ft. Detrick, Frederick, MD		12. REPORT DATE 20 November 1978
		13. NUMBER OF PAGES 22 + 2 figures
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) 26p.		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release – distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Reprints bearing assigned AD number will be forwarded upon receipt.
To be published in TOXICOLOGY AND APPLIED PHARMACOLOGY.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Indomethacin
Hepatic metallothionein
Zinc metabolism
Intestinal lesions

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Altered zinc homeostasis is one of several metabolic sequelae of inflammation wherein zinc is redistributed to the liver from endogenous sources such as plasma. Studies were performed in rats to determine the effect(s) of indo-methacin, a potent anti-inflammatory and ulcerogenic agent, on zinc metabolism. A single sc or ip dose of indomethacin given to fed rats in amounts between 1-10 mg/100 g body weight induced, by 24 hr, profound hypozincemia, enhanced intestinal absorption of zinc, induction of hepatic metallothionein synthesis,

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. (Continued)

and gastrointestinal lesions.   These responses were significantly muted when the drug was administered to fasted rats.   The effects of indomethacin on zinc homeostasis appears to be related to drug-induced enteropathy.   Furthermore, results provide evidence that hepatic metallothionein functions, at least in part, to sequester zinc in pathophysiologic conditions.

ABSTRACT

Altered zinc homeostasis is one of several metabolic sequelae of inflammation wherein zinc is redistributed to the liver from endogenous sources such as plasma. Studies were performed in rats to determine the effect(s) of indomethacin, a potent anti-inflammatory and ulcerogenic agent, on zinc metabolism. A single sc or ip dose of indomethacin given to fed rats in amounts between 1–10 mg/100 g body weight induced, by 24 hr, profound hypozincemia, enhanced intestinal absorption of zinc, induction of hepatic metallothionein synthesis, and gastrointestinal lesions. These responses were significantly muted when the drug was administered to fasted rats. The effects of indomethacin on zinc homeostasis appears to be related to drug-induced enteropathy. Furthermore, results provide evidence that hepatic metallothionein functions, at least in part, to sequester zinc in pathophysiologic conditions.

Alterations in zinc homeostasis characterized by pronounced hypozincemia has been shown to occur in various pathophysiologic conditions and to involve sequestration of zinc by hepatic metallothionein (MT) (Sobocinski et al., 1977, 1978a, 1978b). MT, an inducible pleomorphic low molecular weight protein containing a large number of cysteinyl residues, possesses high affinity for heavy metals such as zinc, mercury, and cadmium (Kojima and Kägi, 1978). Synthesis of this unique metalloprotein appears to be controlled at transcription and translation processes by a zinc-stimulated mechanism (Squibb et al., 1977).

Recently, we have suggested that redistribution of zinc from plasma to liver and enhanced hepatic MT accumulation occur as sequelae of an inflammatory response induced by diverse phlogistic agents which include turpentine, isoproterenol, and cadmium (Sobocinski et al., 1978b) as well as bacterial endotoxin (Sobocinski et al., 1977), bacterial infections (Sobocinski et al., 1978a), and hypersensitivity reactions (Sobocinski et al., 1979). The apparent common denominator relating the induction of hepatic MT to such diverse stimuli appears to be the movement of endogenous zinc into the liver.

In studies conducted in our laboratory, and cited above, a consistent finding has been that the quantity of zinc associated with hepatic MT greatly exceeds the amount lost from the total plasma pool. Although redistribution from body tissues could possibly account for the additional zinc, increased absorption from the intestinal contents may constitute an important mechanism wherein this additional zinc is made available to the liver. An increased intestinal absorption of zinc has recently been demonstrated in cadmium-treated (Winge et al., 1978), infected, and endotoxemic rats (Pekarek and Evans, 1975).

A recent report by Song and Adham (1978) suggests that certain prostaglandins facilitate zinc transport through the intestinal mucosa. Based on the latter observation and the well known role of prostaglandins in inflammation, we postulated that there may be a relationship between the inflammatory response previously noted by us (Sobocinski et al., 1978b), increased intestinal zinc absorption, and subsequent induction of hepatic MT.

In order to test the postulate, we treated rats with indomethacin (IND), a potent inhibitor of prostaglandin synthesis (Vane, 1971), prior to cadmium administration in an attempt to block the inflammatory response and zinc absorption by the intestine. Contrary to expectations, IND treatment alone induced hypozincemia, increased intestinal absorption of zinc, and enhanced MT synthesis. Subsequent studies were performed to obtain information on the pathophysiologic mechanisms involved in drug-induced alterations in zinc metabolism. Evidence was sought concerning the possibility that these alterations were related in some manner to the well known induction of intestinal lesions by IND (Brodie et al., 1970).

MATERIAL AND METHODS

Animals

Male Fisher-Dunning rats (Harlan Industries, Indianapolis, Ind.), weighing 200-250 g, were housed in environmentally controlled facilities as previously described (Sobocinski et al., 1978a). Except as noted in the text, rats were provided food (Wayne Lab-Blox, Allied Mills Inc., Chicago, Ill.) and water ad libitum.

Treatments   :

Indomethacin, 1-[p-chlorobenzoyl]-5-methoxy-2-methyl indole-3-acetic acid (Sigma Chemical Co., St. Louis, Mo.), was dissolved in 47 mM Na_2CO_3 and passed through a 0.45-μm filter (Millipore Corp., Bedford, Mass.) just prior to use. The drug was administered sc or ip on a body weight basis with the amounts and route of administration specified in figure and table legends. Control animals received an equivalent volume of Na_2CO_3 solution (1.0 ml/100 g body weight).

Heavy metal induction of hepatic MT was accomplished by the sc administration of cadmium, as $CdCl_2$ (Fisher Scientific Co., Fair Lawn, N.J.) dissolved in physiological saline (0.3 mg Cd^{++}/100 g body weight). Control animals received an equivalent volume of saline (0.5 ml/100 g body weight).

Tissue Sampling

Rats were killed, at times specified in figure and table legends, after various treatments by exsanguination under halothane anesthesia. Plasma and liver samples were obtained as previously described (Sobocinski et al., 1978a). Selected tissues of the gastrointestinal tract to include portions of stomach, small intestine, and colon were immediately placed in 10% neutral buffered formalin for subsequent histological examination. Tissues were embedded in paraffin, cut in 6-μm sections, stained with hematoxylin and eosin, and examined by light microscopy.

Analytical Procedures

Hepatic MT was isolated by previously published procedures employing acetone fractionation of heat-treated hepatic cytosol (Sobocinski et al, 1978a). The zinc or cadmium content of the isolated

MT, as applicable, was used as a measure of hepatic MT concentration and is expressed as μg metal/ml heat-treated cytosol where 1 ml of cytosol is approximately equivalent to 0.3 g of wet liver weight. Protein concentration of these fractions was not determined since neither Lowry nor biuret methods yield valid results (Weser et al., 1973). In certain instances, MT concentration is expressed as μg protein per unit wet weight of liver with the assumption that 7 g-atoms of metal are bound per 6800 g of protein (Kojima and Kägi, 1978).

The effect of IND on zinc absorption from the intestinal tract was determined after po administration of 20 μCi carrier-free ^{65}Zn (New England Nuclear, Boston, Mass.) by the method of Pekarek and Evans (1975). Plasma and MT zinc, cadmium concentrations were determined by atomic absorption spectrophotometry (Pekarek et al., 1972).

Possible drug-induced endotoxemia and/or bacteremia were evaluated by Limulus Amebocyte Lysate test, LAL (Microbiological Associates, Walkersville, Md.) and blood cultures, respectively. The LAL test was performed on plasma obtained by centrifugation of blood collected in the pleural cavity after transsection of the vena cava (Sobocinski et al., 1978a) and on plasma obtained from portal venous blood. Precautions, cited by the LAL manufacturer in test methodology, were taken to avoid the influence of nonspecific test inhibitors possibly present in blood products.

Statistics

Significance of differences between group means was determined by one-way analysis of variance. A P value $\leq$ 0.05 was considered significant.

RESULTS

Results of initial experiments performed to determine the effect of cadmium and IND administration on plasma zinc and hepatic MT concentrations are shown in Table 1. Both substances induced hypozincemia and enhanced hepatic MT accumulation when separately administered. In rats administered IND, 24 hr prior to cadmium, cadmium induced a further increase in MT concentration when compared to either values obtained when Cd or IND was administered alone. Pretreatment with IND appears to enhance Cd clearance from plasma and its sequestration in MT since the data shown in Table 1 indicate that approximately twice as much Cd is present in hepatic MT in IND-pretreated rats compared to those administered Cd alone. Increased movement of Cd from plasma to liver in IND-treated rats compared to those receiving Cd alone is further indicated by the lower plasma Cd concentrations in IND-treated rats. Very small amounts of MT were found in untreated control rats and ranged from approximately 20-60 μg/dl between experiments.

Subsequent experiments were performed to examine further the effect of IND on zinc homeostasis. The relationship between the extent of plasma zinc depression and hepatic MT accumulation at various drug doses is shown in Figure 1. An inverse relationship exists between plasma zinc depression and MT accumulation over the dose range 1-5 mg/100 g body weight. No effect of the drug on these parameters was found for doses equal to or less than 1.0 mg/100 g body weight. For doses between 1 and 5 mg/100 g body weight a nearly linear log-dose response existed with no significant differences observed between

responses obtained with 5 and 10 mg.

Intestinal zinc absorption was measured 1 hr after a po pulse dose of ^{65}Zn administered 16 hr after drug treatment (Table 2). When administered at a dose of 10 mg/100 g body weight, IND induced a significant 3-fold increase in zinc absorption from the intestinal tract when compared to untreated drug controls.

In order to obtain information as to whether the observed drug effects on zinc homeostasis were directly attributable to the drug or to some drug-related side effect such as enteropathy (Kent et al., 1969; Brodie et al., 1970), experiments using the 10-mg dose regimen were repeated in both fed rats and rats starved for 24 hr. Previous work has demonstrated that drug-induced enteropathy is effectively prevented by prior fasting of recipient rats (Brodie et al., 1970). The data presented in Table 3 demonstrate that IND induced plasma zinc depression and MT accumulation is dependent on dietary treatment and can be significantly prevented by prior fasting.

Neither endotoxemia nor bacteremia could be demonstrated in fed rats given the 10 mg of IND.

Histological examination of various gastrointestinal tissues was performed in an attempt to correlate further alterations in zinc homeostasis with the distribution and severity of drug-induced lesions at selected drug doses (1, 2.5, and 10 mg/100 g body weight). Rats that were fed normally and received 10 mg IND sc had multiple, erosive to often-ulcerative lesions in the stomach, small intestine, and colon. In the small intestine, there was abrupt, deep ulceration which frequently extended into the submucosal tissues. This ulceration was accompanied by an acute, necrotizing process that resulted in

disruption of the muscularis externa and a cellular inflammatory
infiltrate of the serosa and peritoneum (Fig. 2A). These segmental
lesions were sharply delineated from the adjacent normal intestinal
tissue. Although ulceration was less common in the stomach and colon,
the affected segments of these organs contained an intense
polymorphonuclear infiltrate that frequently obliterated portions
of the mucosa, submucosa, and muscularis externa. Large clusters of
bacterial organisms were consistently observed in the lumen adjacent
to the affected colonic segments (Fig. 2B).

Fasted rats that received 10 mg IND sc had moderate to severe
lesions that were primarily confined to the large intestine. The
colitis was characterized by segmental mucosal erosions and an
occasional ulceration with a polymorphonuclear infiltrate involving
most of the colonic wall. Other than the absence of serosal lesions
or peritoneal involvement, these colonic lesions were quite similar
to those observed in the fed animals, but were less numerous, with
smaller segments of the colon affected. There was only an
occasional change observed in the small intestine. Within short
segmental lesions, there was mild sloughing of the intestinal villi
with distension of the lamina propria and submucosa by edema and a
polymorphonuclear infiltrate that extended into the serosa (Fig. 2C).

Fed rats that received 2.5 mg IND had erosive to ulcerative
lesions in the colon and to a lesser extent, the small intestine.
The intestinal lesions were again segmental in nature with severe
necrosis extending from the ulcerated mucosa into the submucosa, with
some disruption of the muscle layers and occasional cellular

infiltration of the serosa (Fig. 2D). Although these lesions are histologically similar to those described in the 10-mg fed rats, the affected segments of the intestine were generally much shorter and less numerous than in the high-dose animals.

Fed rats that received 1.0 mg/100 g body weight had no gastrointestinal lesions attributable to IND treatment. Control animals that received sodium carbonate had essentially normal tissues.

DISCUSSION

Results presented in this study demonstrate that IND-induced alterations in zinc homeostasis occur, at least in part, as a consequence of drug-induced enteropathy. Further, results contribute to an increasing volume of evidence documenting the involvement of hepatic MT in the sequestration of zinc which occurs in various pathophysiologic conditions (Sobocinski et al., 1977, 1978a, b, c, 1979) as well as in normal zinc homeostasis (Richards and Cousins, 1975).

Cadmium and zinc induction of de novo hepatic MT synthesis is well known and appears to occur via metal-stimulated mechanisms with regulation at translation as well as transcription (Squibb et al., 1977). In the present study, IND has been shown to increase absorption of intestinal zinc by as yet unknown mechanism(s). Possibilities include drug-induced (a) detrimental effects on normal regulatory mechanisms involved in zinc transport in mucosal cells (Richards and Cousins, 1975) or (b) physiologic changes involving gut motility and excretion rate. Some evidence suggesting that cadmium administration

to rats enhances zinc mobilization by affecting excretion has recently been presented by Winge et al. (1978). Other substances, which have been shown to induce MT synthesis, such as bacterial endotoxin and leukocytic endogenous mediator (LEM) (Sobocinski et al., 1977) also increase intestinal absorption of zinc (Pekarek and Evans, 1975, 1976). An increase in the amount of zinc available to the liver apparently can provide the stimulus for de novo synthesis of MT (Richards and Cousins, 1975, Squibb et al., 1977). It appears likely that similar mechanisms may be involved in IND-induced hepatic MT accumulation. However, the relative contribution of redistribution of endogenous zinc and increased intestinal zinc absorption to MT synthesis remains unclear.

The present findings have led us to postulate that hypozincemia occurs, in many pathophysiologic conditions involving redistribution of endogenous zinc, concomitant with induction of hepatic MT. Once MT synthesis is initiated, for example, by increased availability of zinc to the liver through processes which include redistribution of tissue zinc and enhanced gut absorption, MT effectively scavenges zinc from available body pools such as the plasma. No evidence is available which would indicate that the relatively small amount of zinc available in the plasma pool is sufficient to stimulate MT synthesis.

Although a previous report (Song and Adham, 1978) suggested that IND prevents intestinal zinc absorption by inhibiting prostaglandin synthesis our results indicate that the drug enhances absorption. Use of fasted rats by these authors may explain the apparent discrepancy, since fasting prior to drug administration prevents

enteropathy. Other differences in experimental design, such as time and route of drug administration could also be involved. These authors did, however, note an increase in jejunal zinc content in rats 5 hr after a comparable dose of IND (10 mg in 100-150-g rats).

Although there was no attempt to quantify histologic results in this study, it was apparent during evaluation of the lesions, that there was a relationship between the severity of the IND-induced small intestine lesions and hypozincemia. For example, plasma zinc was least altered in fasted rats (10 mg IND) that generally had mild changes in the small intestine, whereas in rats receiving 2.5 mg, there was more extensive intestinal involvement and plasma zinc was significantly depressed. Similarly, plasma zinc levels were lowest in the high-dose, fed animals where the enteropathy was most severe. In contrast, the extent of gastric and colonic involvement appeared to have little effect on zinc homeostasis.

The pathogenesis of the IND-induced lesions has not been defined. The segmental necrosis with only a mild polymorphonuclear response in the small intestine is suggestive of an ischemic mechanism. However, the absence of significant vascular involvement and the presence of an intense cellular infiltrate in the stomach and colon lesions lends little support to an ischemic etiology.

It is unknown what role the endogenous bacteria play in the development of the gastrointestinal lesions and altered zinc homeostasis. In the present study, there was an apparent proliferation of bacterial organisms in the gut lumen adjacent to the necrotic, eroded or ulcerated segments. These bacterial clusters were not observed in unaffected portions of the gastrointestinal tract or in

control animals. These observations are in agreement with other investigators that report an intestinal flora overgrowth in IND-treated rats (Kent et al., 1969). It is reported that pretreatment with antibiotics or the use of gnotobiotic rats prevents the development of IND-induced lesions (Kent et al., 1969). Previous attempts to explain these findings (Del Soldato and Meli, 1978) indicated that excessive production of β-glucuronidase, attributable to proliferation of intestinal flora, enhanced deconjugation of IND, and subsequent ulceration due to free IND or a metabolite.

It is emphasized in the present study that regardless of treatment, the colon appears to be the portion of the gastrointestinal tract that is most susceptible to the effects of IND. Other investigators emphasize lesions occurring in the stomach and mid to terminal small intestine of IND-treated rats. This discrepancy in the distribution of lesions can in part be explained by possible differences in strain susceptibility (Fang et al., 1977).

Because of our previous observations that endotoxemia (Sobocinski et al., 1977) and various bacterial infections (Sobocinski et al., 1978a) induce hypozincemia and enhanced hepatic MT accumulation, it was important in the present studies to address the possibility that IND effects on zinc homeostasis were attributable to either drug-induced leakage of enteric endotoxin and subsequent endotoxemia or sepsis arising from intestinal lesions. Neither condition could be demonstrated at 24 hr in fed rats given the highest dose of IND (10 mg/100 g body weight).

In summary, results of our studies provide strong evidence that the action of IND on zinc homeostasis is related to drug-induced

enteropathy and that hepatic MT is intimately involved in the
redistribution of zinc which occurs in various pathophysiological
states. With a metal binding capacity of 7 g-atoms per mole (Kojima
and Kägi, 1978) and a relatively short half-life (Feldman and Cousins,
1976; Sobocinski et al., 1978a), hepatic MT accumulation constitutes
an effective means for achieving short-term redistribution of
endogenous zinc.

Acknowledgments

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TABLE 1

EFFECT OF INDOMETHACIN (IND) PRETREATMENT ON CADMIUM-INDUCED CHANGES IN

PLASMA ZINC CONCENTRATION AND HEPATIC METALLOTHIONEIN CONTENT

Group (n)	Treatment[a]	Plasma		MT			
		Zn^b (μg/dl)	Cd^b (μg/dl)	Zn^b (μg/dl)	Cd^b (μg/dl)	g-atom ratio (Cd/Zn)	Mean total Concentration[c] (μg/g)
1 (5)	None	116 ± 2	< 2	18 ± 4	< 1	--	9
2 (5)	Cd	48 ± 3[d]	18 ± 2	81 ± 16	416 ± 38	3.0	160
3 (5)	IND	31 ± 2[d,e]	< 2	481 ± 76[d,e]	< 1	--	239
4 (5)	IND + Cd	23 ± 2[d,e]	8 ± 1[e]	460 ± 32[d,e]	760 ± 47[e]	1.0	447

[a] IND was administered ip (10 mg/100 g body weight). Cadmium, as $CdCl_2$ (0.3 mg Cd^{++}/100 g body weight) was administered sc. In combined treatments, IND was administered 24 hr prior to cadmium. All rats were killed 5 hr after the time of cadmium administration; 29 hr after indomethacin.

[b] Values are means ± SE. Values for MT are expressed in terms of concentration found in MT fractions isolated from specified volume of heat-treated hepatic cytosol (Sobocinski et al., 1978a).

[c] Calculated on a liver wet weight basis with the assumption that 7 g-atoms of various metals are bound per molecular weight of 6800 (Kojima and Kägi, 1978).

[d] Significantly different vs. group 1, $P \leq 0.001$.

[e] Significantly different vs. group 2, $P \leq 0.001$.

TABLE 2

EFFECT OF INDOMETHACIN (IND) ON

INTESTINAL ABSORPTION OF ^{65}Zn

Group (n)	Treatment[a]	Carcass ^{65}Zn[b] (% absorbed)
1 (10)	IND	29.5 ± 6.6[c]
2 (10)	Control	9.4 ± 1.7

[a] IND was administered sc (10 mg/100 g body weight). Control rats received an equivalent volume of Na_2CO_3 solution (1 ml/100 g body weight) used to solubilize IND. Carrier-free ^{65}Zn (20 µCi) in physiological saline was administered po (0.5 ml) 16 hr after IND or Na_2CO_3 administration. Rats were killed 1 hr after ^{65}Zn administration.

[b] Values are means $\pm$ SE. Absorption, expressed as a percent of total ^{65}Zn-radioactivity found in carcass plus intestine, was calculated according to the method of Pekarek and Evans (1975).

[c] Significantly different, $P < 0.01$.

TABLE 3

EFFECT OF INDOMETHACIN (IND) ON PLASMA ZINC CONCENTRATION AND

ACCUMULATION OF HEPATIC METALLOTHIONEIN (MT) ZINC IN FED AND FASTED RATS

Group (n)	Treatment[a]		Plasma[b]	MT[b,c]
	Diet	Substance Administered	(μg/dl)	(μg/dl)
1 (16)	Fed	IND	40 ± 3[d]	242 ± 15[d]
2 (10)	Fed	Na_2CO_3	108 ± 5	28 ± 5
3 (15)	Fed	None	113 ± 3	45 ± 9
4 (10)	Fasted	IND	83 ± 3[d]	94 ± 12[e]
5 (10)	Fasted	Na_2CO_3	108 ± 3	66 ± 6
6 (10)	Fasted	None	109 ± 2	53 ± 3

[a] Food was removed 24 hr prior to sc injection of either IND

(10 mg/100 g body weight) dissolved in Na_2CO_3 or an equivalent amount

of Na_2CO_3 solution. Food was withheld during the subsequent 24 hr

period. All rats were sacrificed 24 hr after the time of injection.

[b] Values represent means $\pm$ SE.

[c] Values expressed in terms of concentration found in metallothioneins

isolated from specified volume of heat-treated hepatic cytosol

(Sobocinski et al., 1978a).

[d] Significantly different; $P \leq 0.001$ (group 1 vs. 2-6 and group 4 vs.

5 or 6).

[e] Significantly different; $P \leq 0.05$ (group 4 vs. 6).

Figure Legends

Fig. 1 . Effect of various amounts of indomethacin on plasma (●)

and hepatic (□) metallothionein (MT) zinc concentrations at

24 hr. Horizontal bars indicate control values $\pm$ SE for 10

rats, plasma zinc (top) and hepatic MT (bottom). Points are

means $\pm$ SE of 9-10 rats. [*]P < 0.001 vs. controls.

Fig. 2A Section of small intestine from fed rat administered IND sc,

10 mg/100 g body weight, showing a long, ulcerated, and

necrotic segment of intestine with only a portion of the

submucosa remaining and disruption of the muscularis externa.

Inflammatory cell infiltrate extends into peritoneal adipose

tissue. x 70.

Fig. 2B Section of colon from fed rat administered IND sc, 10 mg/100 g

body weight; eroded segment of colon with intense

polymorphonuclear infiltration in the mucosa and the edematous

submucosa. Small clusters of bacteria are present in the

lumen adjacent to the involved segment. x 70.

Fig. 2C Section of small intestine from fasted rat administered IND

sc, 100 mg/100 g body weight, showing mild sloughing of

intestinal villi with distension of the lamina propria and

submucosa by edema and polymorphonuclear infiltration. x 160.

Fig. 2D Section of small intestine from fed rat administered IND sc,

2.5 mg/100 g body weight; short ulcerated segment with

disruption of the muscularis externa and a polymorphonuclear

cell infiltrate in the serosa. x 70.